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Effects of methylprednisolone alone and combined with erythropoietin on the antioxidant system in rats with induced acute spinal cord injury

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Abstract

Aim: This experimental study was carried out to biochemically compare the effects of methylprednisolone (MP) alone and in combination with human recombinant erythropoietin (EPO) on malondialdehyde (MDA), superoxide dismutase (SOD) and catalase.

Material and Methods: Twenty-four adult male Sprague-Dawley rats were randomly divided into three groups. Spinal cord injury (SCI) was created after laminectomy in all three groups. No treatment was given to the trauma group. In the MP group, 30mg/kg fast (within 15 minutes), and after 45 minutes, intraperitoneal MP was administered in 4 equal doses of 5.4 mg/kg continuously within 23 hours. In the EPO + MP group, 1000IU/kg intraperitoneal EPO was given in addition to the MP given in the same way. MDA, SOD and catalase levels were compared between the groups in the spinal cord samples taken from all rats at the 24th hour after the operation.

Results: Catalase level was higher in both the MP group and the EPO + MP group compared with the trauma group ($p = 0.006$, $p = 0.001$; respectively). Compared to the trauma group, the SOD level was higher only in the EPO + MP group ($p = 0.006$). Both catalase and SOD levels were higher in the EPO + MP group compared to the MP group ($p = 0.006$, $p = 0.005$; respectively). There was no difference between the groups in terms of MDA ($p = 0.183$).

Discussion: These results show that EPO given in addition to MP in SCI can be used effectively against oxidative damage.

Keywords

Spinal cord; Methylprednisolone; Erythropoietin; Malondialdehyde; Superoxide dismutase; Catalase

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Introduction

Spinal cord trauma is a condition, which results in mortality as well as causes severe disability. Motor vehicle accidents, falls, violence, sports and occupational accidents are among the main causes of spine and spinal cord injuries [1]. In Turkey, between 1600-2000 cases of serious spinal injuries are reported annually [2]. Primary damage occurs as a result of mechanical impact in spinal cord injury. The damage to the spinal cord tissue secondary to mechanical injury increases over time, causing clinical worsening. The primary goal of clinical treatment is to stop or slow down this secondary damage cascade. Necrosis and apoptosis were initially identified as two main mechanisms of cellular death following SCI [1].

Normally, harmful effects of free radicals that form in the mitochondria are eliminated by antioxidant systems. If the amount of free radicals excessively increases, the antioxidant systems become insufficient, leading to cellular death. The superoxide radical (O_2^-), which forms in the mitochondria, converts superoxide dismutase (SOD) into hydrogen peroxide (H2O2), and the catalase enzyme converts hydrogen peroxide into H2O and O2. Thus, the tissue is protected from damage, preventing the formation and spreading of reactive O2 radicals [3]. However, these antioxidant mechanisms rapidly decrease following a trauma [4]. This causes more free radicals to appear. Following spinal cord injury, hemoglobin catalyzes the peroxidation of iron membrane phospholipids that are released by ferritin or transferrin. Eventually, the membrane breaks down and the cell dies. In addition, free oxygen radicals disrupt the blood–spinal cord barrier through endothelial damage, causing the accumulation of harmful substances in the injury site. The central nervous system is prone to free radical damage due to low activity of SOD and catalase [3,4]

These free radicals cause the oxidation of proteins and lipids in the cell membrane, impairing the membrane fluidity and ionic gradient. Malondialdehyde (MDA), the most prominent product of lipid peroxidation, is also used to determine lipid peroxidation. MDA diffuses easily, cross-binds to lipids and proteins in the membrane structure, and disrupts permeability by changing the specific properties of the membrane [5].

Numerous chemical agents have been used in order to protect tissues against the damage occurring after spinal cord trauma. Some agents have decreased tissue damage, while others have increased functional improvement. Although corticosteroids are one of the drugs used for this purpose, it is thought that their antioxidant features are more prominent rather than glucocorticoids-receptor association in the inhibition of lipid peroxidation. In a study, improvement in sensory and motor function was demonstrated with methylprednisolone (MP) used after spinal cord injury [6]. Another agent used in spinal cord injury is recombinant human erythropoietin (EPO), which is a hematopoietic growth factor that stimulates proliferation and differentiation in erythrocyte precursor cells. Studies have shown that erythropoietin and erythropoietin receptors are present in the central nervous system. In vivo and in vitro neural damage model studies searched antiapoptotic effect of erythropoietin, and it was reported to inhibit apoptosis, decrease inflammation, regulate excitotoxicity and increase neural proliferation [7]. In the light of this information, in a study comparing EPO and interleukin-6 in rats with induced acute SCI, EPO was shown to be effective [8].

In our study, we aimed to search studies in the literature and biochemically compare the effects of MP alone and combined with EPO on SOD, catalase, and MDA in rats with induced SCI.

Material and Methods

A total of 24 adult Sprague-Dawley rats produced in the Production and Purification Laboratory of the Istanbul Universitesi, Department of Experimental Animals Biology and Biomedical Application Techniques were used in this study. The rats weighed between 280-300 g. The animals were randomly divided into three groups of eight rats each. After the rats were anesthetized with 60 mg/Kg intraperitoneal ketamine hydrochloride and 10 mg/Kg intraperitoneal xylazine, the posterior part of the 8-10th spinal bones was removed and a non-dural trauma was created for 60 seconds using the Yasargil aneurysm clip at a pressure of 0.7 N. Hereafter, the groups were created as follows: i) Trauma group: did not receive any treatment ii) MP group: following 30 mg/Kg rapid MP administration (within 15 minutes), after 45 minutes, intraperitoneal MP was constantly given at 4 equal doses of 5.4 mg/Kg within 23 hours iii) EPO+MP group: following 30 mg/Kg rapid MP administration (within 15 minutes), after 45 minutes, a combination of MP + 1000 IU/Kg EPO was constantly administered intraperitoneally at 4 equal doses of 5.4 mg/Kg within 23 hours.

Following the operation, the rats were anesthetized with 60 mg/Kg intraperitoneal ketamine hydrochloride and 10 mg/Kg intraperitoneal xylazine, and the animals were then sacrificed with the perfusion of 100 mg/Kg sodium-pentothal. Spinal cord samples were collected and the levels of MDA, SOD and catalase were measured in these samples.

This study was carried out in accordance with the "Universal Declaration of Animal Rights", the "European Convention on the Protection of Vertebrates for Experimental and Other Scientific Purposes (Council of Europe ETS 123)", the "Handbook for the Care and Use of Laboratory Animals (National Research Council, USA)", "Animal Protection Law (No. 5199)" and the "Regulation on the Working Procedures and Principles of Animal Experiments Ethics Committees" prepared by the Ministry of Environment and Forestry published in the Official Gazette (dated February 15, 2014 and No. 28914).

Preparation of Tissue Homogenate

Medulla spinalis was weighed and mixed with a 0.1 M phosphate buffer at a ratio of 1:9. The mixture was homogenized on the ice at 10.000 rpm for 1 second with a homogenizer (MICCRA GmbH Grißheimer Weg 5 79423 Heitersheim/Germany). The homogenized samples were centrifuged at 5000 g and +4oC in a refrigerated centrifuge. Protein determination was made in the supernatants of the homogenized samples in the Siemens Advia device (Block Scientific, 22 Sawgrass Drive Bellport, NY 11713 USA). Tissue protein levels were determined using the BOS protein measurement kit.

Measurement of Catalase Level:

Catalase level was spectrophotometrically evaluated, and its absorbance was read at 520 nm (Bioxytech catalase-520, catalog no: 21042 Oxis Research Product kits, USA). All

processes were carried out at room temperature. The results were expressed as Unite/g protein.

Measurement of Malondialdehyde (MDA) Level:

MDA levels were studied with the spectrophotometric methods (Bioxytech MDA-5861 catalog no: 21044-Oxis Research Product kits, USA). We aimed to measure free MDA levels following hydrolysis with the MDA-586 method. The results obtained in the spectrophotometry at 586 nm were expressed as µmol/g tissue.

Measurement of Superoxide Dismutase (SOD) Activity:

SOD (EC 1.15.1.1.) activity was measured on the basis of the reduction of nitroblue tetrazolium (NBT) by superoxide produced through the xanthine-xanthine oxidase system. The colorimetric plate where the reactions occurred was measured at 560 nm with the BioTek ELISA reader (BioTek Instruments, Kocherwaldstr. 34 D-74177 Bad Friedrichshall, Germany). The results were expressed as U/mg wet tissue.

Statistical Analysis

The data obtained in the study were evaluated using SPSS 20 (Statistical Package for Social Sciences) for Mac OS software. Descriptive statistics were expressed as median, 25th and 75th percentiles. When evaluating the study data, since the parameters were non-normally distributed, the Kruskal-Wallis test was used in the comparison of the variables between the groups, and the Mann-Whitney test was used to determine the group, which caused the difference. The level of statistical significance was set at $p<0.05$ for the Kruskal-Wallis test, and $p<0.016$ for the Mann-Whitney U test with Bonferroni correction.

Results

MDA parameter:

According to the results of the Kruskal-Wallis one-way variance analysis performed for multiple comparisons between the groups, no significant difference was found between the groups in terms of MDA parameter at 95% confidence interval and $p<0.05$ significance level ($p=0.183$).

On the other hand, there were statistically significant differences between the groups in terms of catalase and SOD ($p<0.001$ and $p=0.004$; respectively) (Table1). The comparison was made between paired groups to determine the groups, which caused the difference. Since there were three groups subjected to paired comparisons, (Group 1: Trauma-MP, Group 2: Trauma-EPO+MP and Group 3: MP-EPO+MP), the statistical significance level was taken as 0.05/3=0.016 with Bonferroni correction (Table 2).

Catalase parameter:

There was a statistically significant difference between the Trauma and MP groups (Z=-2,731; p= 0.006). The median catalase level was higher in the MP group. There was a statistically significant difference between the Trauma and EPO+MP groups (Z=-3,361; p= 0.001). The median catalase level was higher in the EPO + MP group. There was a statistically significant difference between the MP and EPO+MP groups (Z=-2,731; p= 0.006). The mean catalase level was higher in the EPO+ MP group. The differences between the groups in terms of catalase level are shown in Figure 1.

For SOD parameter:

No statistically significant difference was found between the Trauma and MP groups in terms of SOD parameter (Z=-1.155; p= 0.248). There was a statistically significant difference between the Trauma and EPO+MP groups (Z=-2,731; p= 0.006). The median SOD level was higher in the EPO+MP group. There was a statistically significant difference between the MP and EPO+MP groups (Z=-2,836; p= 0.005). The median SOD level was higher in the EPO+MP group. Differences between the groups in SOD levels are shown in Figure 2. An overall comparison of enzyme levels between the groups is given in Figure 3.

Table 1. Variance analysis of the Untreated Trauma, Methylprednisolone and Erythropoietin+ Methylprednisolone groups

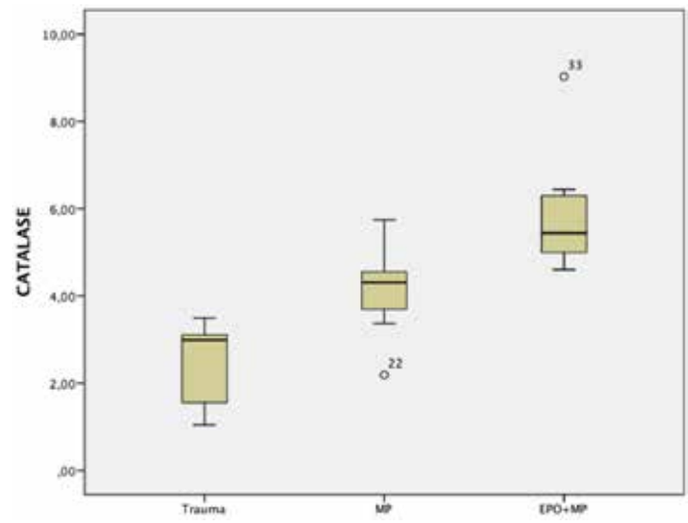
	Median (%25-75 Percentile)			Chi-square*	p
	Trauma	MP	EPO+MP		
MDA (µmol/mg protein)	11.84 (9.89-12.50)	9.30 (5.27-11.20)	7.58 (4.45-12.14)	3.39	0.183
Catalase (U/mg protein)	2.98 (1.42-3.13)	4.30 (3.53-4.60)	5.44 (4.99-6.36)	16.82	<0.001
SOD (mU/mg protein)	6.98 (4.51-8.75)	9.30 (4.59-10.44)	16.63 (13.90-19.37)	11.08	0.004

*Kruskal -Wallis Test, MDA= Malondialdehyde, SOD= Superoxide dismutase, MP= Methylprednisolone, EPO= Human recombinant erythropoietin, p<0.05 statistical significance level

Table 2. Comparison of ROS parameters between the grroups

Groups	ROS	Z*	p
Trauma-MP	MDA	-1.687	0.092
	Catalase	-2.731	0.006
	SOD	-1.155	0.248
Trauma-EPO+MP	MDA	-1.471	0.141
	Catalase	-3.361	0.001
	SOD	-2.731	0.006
MP-EPO+MP	MDA	-0,264	0,792
	Catalase	-2.731	0.006
	SOD	-2,836	0,005

*Mann-Whitney U Test, MDA= Malondialdehyde, SOD=Superoxide dismutase, MP= Methylprednisolone, EPO=Human recombinant erythropoietin, ROS= reactive oxygen species, p<0.016 statistical significance level



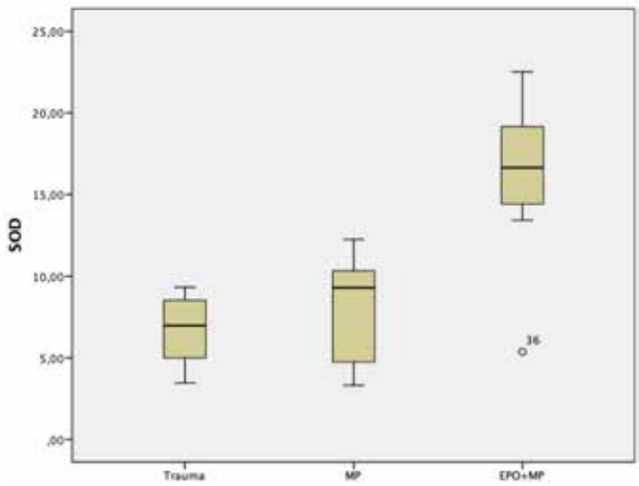


Figure 2. Superoxide dismutase (SOD) level differences between groups, SOD=mU/mg proteine

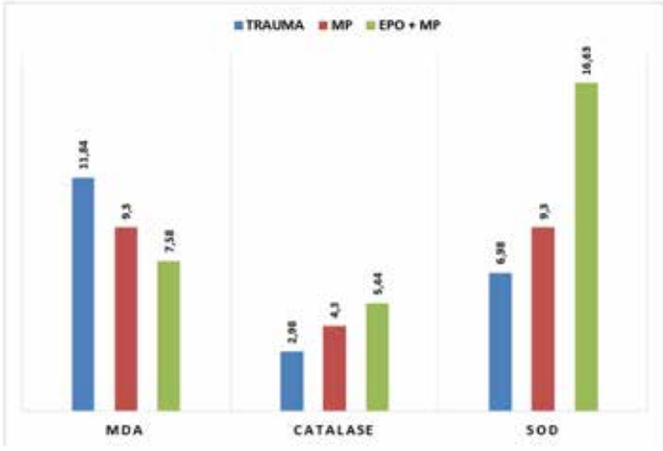


Figure 3. Comparison of the enzyme levels between the groups

Discussion

Studies that have attempted to explain the etiology and pathogenesis of SCI have found that increased formation of reactive oxygen species (ROS) and resultant oxidative stress are crucial events related to SCI. Neurons and glia in the central nervous system, including the spinal cord tend to be subjected to oxidative and electrophilic stress because of numerous factors such as high polyunsaturated fatty acids content, high rate of oxidative metabolic activity, intense production of reactive oxygen metabolites, and relatively low antioxidant capacity. In fact, oxidative stress is considered a distinctive feature of the secondary phase of SCI. Therefore, alleviating oxidative stress is seen as an important step in the treatment of SCI [9].

Since each market has its own limitation in predicting oxidation in biological systems, it is recommended to use at least two biological markers [9]. In the present study, we biochemically investigated the effects of using MP alone or combined with EPO on MDA, SOD and catalase in rats with induced spinal cord trauma. As the most important and remarkable finding of our study, adding EPO to MP was more effective compared to administration of MP alone.

In the present study, catalase level was higher in the MP group compared to the Trauma group. Similarly, in a study on rabbits with induced SCI, catalase level was found to be higher in the animals given MP than the control group [10]. The role of MP in the antioxidant system has also been investigated in injuries other than SCI [11,12]. In one of these studies, the effect of MP on the glomerular the antioxidant system was examined in rats with glomerular damage, and catalase activity was found to increase with MP application [12]. It has been shown in other studies that administration of high-dose MP immediately after SCI exerts neuroprotective effects [13]. Although the underlying mechanisms have not yet been clarified, preventing lipid peroxidation, free radical formation and edema at the injury site are among the prominent mechanisms [14]. Thus, treatment with MP considerably decreases tissue necrosis and paralysis following injury [14]. In our study, the higher levels of catalase in the MP group are consistent with the studies in the literature.

In our study, no statistically significant difference was found between the Trauma and MP groups in terms of the SOD parameter. This result does not seem compatible with the studies in the literature. In a study comparing curcumin and MP, SOD levels were found to be higher in the curcumin group. However, in that study, SOD levels were higher in both curcumin and MP groups compared to the control group, which was not given any drug [15]. In addition, MP, which was given following damage in the glomerular cells of rabbits, was shown to decrease SOD level [12].

Various combinations with MP have been tried for the treatment of SCI. Studies using melatonin [11] and placenta-derived mesenchymal stem cells [10] in addition to MP are examples of combination therapies. As specified, in our study, EPO was used in addition to MP.

Although EPO's mechanism of action on the central nervous system is unclear, according to current knowledge, its hematopoietic and neuroprotective properties act through different signaling systems. The receptor that produces the neuroprotective effect and the receptor that creates the hematopoietic effect are different from each other [16]. In a study by Grosso et al. [16], it was shown that erythropoietin and erythropoietin receptors increased in the neurons, glial cells and vascular epithelium of rats subjected to trauma, this increase started at the post-traumatic 8th hour and peaked on the 8th day, gradually decreased, and fell considerably 2 weeks after the trauma. In vivo experimental studies have demonstrated that decreased neuronal damage in focal ischemia, traumatic brain injury, inflammation, spinal cord injury and subarachnoid hemorrhage [17]. For this purpose, EPO was used in the treatment of 13 patients with acute paralysis, and very good results were obtained in the one- month follow-up of these patients [18].

Studies have shown that EPO provides efficient protection against ischemia-reperfusion damage in several tissues and organs including the brain [19] and heart [20]. It has been reported that the activity of enzymes such as SOD and catalase that directly or indirectly protect the erythrocyte membrane from peroxidative threat in hungry animals with low blood levels of EPO decreased due to hunger and returned to normal levels following EPO [21]. Similarly, in another study investigating the effectiveness of EPO, it was observed that EPO administered

with melatonin following renal ischemia in rats, increased the levels of SOD and catalase [22]. Yazihan et al. [23] stated that treatment with EPO increased catalase level and EPO significantly decreased oxidative damage following SCI induced in rats. In our study, catalase and SOD levels were higher in the EPO+MP group compared to the Trauma group, in parallel with the findings reported in the literature.

Polyunsaturated fatty acids of membrane phospholipids in the damaged cells in the case of injury can be oxidized by themselves or by binding of oxidation products and turn into peroxide derivatives. MDA, one of these products, is used to determine the level of lipid peroxidation [8]. In our study, no difference was found among the three groups in terms of MDA. However, unlike our study, previous studies showed that MP decreased lipid peroxidation by decreasing MDA level [10,15]. This difference might result from the dose of MP administered per Kg, the duration and regimen of treatment. Differences between the studies might also be caused by other factors.

Protection against secondary injury in acute spinal cord injury is called neuroprotection. For this purpose, many medical and surgical approaches such as drug treatments, correction of tissue oxygenation, removal of spinal cord compression and stabilization of the spine are tried. Numerous studies on pharmacological protection in spinal cord injury have been conducted in the last two decades, but none of these have been standard treatments for use in humans. Secondary injury is a process that begins within minutes to hours and continues for weeks, following the primary injury. Primary injury can be prevented only by preventive measures. The aim of research on secondary injury is to find and use pharmacological agents and precautions to protect the neurons in the lesion site, to increase their durability or to stop the pathological processes that would harm them. Modern pharmacological treatment protocols that are aimed to decrease progressive neuronal damage and minimize the neurological sequel that occurs. With our current information, dose and duration management should be studied with larger series in order to routinely use erythropoietin in neural trauma management.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

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